Expression and Function of MicroRNAs in Heart Disease

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Abstract: microRNAs (miRNAs) are powerful, recently recognized regulators of gene expression. miRNAs modulate virtually all aspects of cardiac biology, from cardiac specification and development to cardiomyocyte survival and hypertrophy. Expression profiling of experimental and human heart disease has shown that miRNA expression is altered in heart disease, and miRNA expression signatures may be useful biomarkers for heart disease diagnosis and prognosis. Mechanistic studies have revealed how miRNAs contribute to heart disease pathogenesis. Here we review the expression and function of miRNAs in heart disease.

Keywords: microRNA, heart failure, hypertrophy, proliferation, apoptosis, fibrosis, angiogenesis, arrhythmia.

INTRODUCTION

The pathogenesis of heart failure is complex and involves disruption of normal mechanisms that regulate cardiomyocyte gene expression, growth, survival, and function. Cardiac interstitial cells and vascular cells also actively participate in the disease process, resulting in altered myocyte-non-myocyte signaling, cardiac fibrosis, and decreased vascular density. Despite extensive research into the pathogenesis and treatment of heart failure, few novel therapeutic approaches have proven to be clinically applicable.

Recently, microRNAs (miRNAs) have elicited substantial interest as therapeutic targets for heart failure. miRNAs are ~22 nucleotide small RNAs that negatively regulate expression of other genes by reducing mRNA stability and/or translation [1, 2]. Regulatory specificity is encoded by partial sequence complementarity between a miRNA and its mRNA targets. Interactions between miRNA positions 2 to 8 (the "seed" sequence) and complementary regions within the 3' UTR of mRNA targets (the "seed match" sequence) are thought to be particularly important for determining miRNA targets [3]. Based on miRNA-mRNA sequence complementarity and its conservation through evolution, computational algorithms have been developed by several groups to predict genes regulated by specific miRNAs ("miRNA targets"; reviewed in [4, 5]).

This review will highlight the roles of miRNAs in cellular processes closely linked to human heart disease. For general background on miRNA biogeneses and and regulatory mechanisms, readers are referred to several recent reviews [1, 2, 6, 7-12].

MIR EXPRESSION IN NORMAL AND DISEASED HEART

Profiling of miRNA expression in normal tissues has revealed that a subset of miRNAs are expressed selectively in striated muscles [13, 14]. Among these are miR-1 and miR-133, two independent miRNAs that generated from the same primary transcript. miR-1 and miR-133 are progressively upregulated during development and peak in the adult heart [15]. Selective expression of these miRNAs in striated muscle is driven by serum response factor (SRF) and MEF2, key transcriptional regulators of cardiac muscle gene expression [16, 17]. Interestingly, SRF is itself negatively regulated by miR-133, creating a negative feedback loop [18]. Another interesting point is that while miR-1 and miR-133 are expressed from the same primary transcript, miR-1 levels are roughly 10-fold higher than miR-133 levels [19], clearly indicating important regulation at the level of miRNA biogenesis or stability. A second group of miRNAs expressed in a myocyte-selective pattern are the "myomirs" miR-208a, miR-208b, and miR-499, miRNAs embedded within introns of myosin heavy chain genes *Myh6*, *Myh7*, and *Myh7b*, respectively [20]. These miRNAs play key roles in regulating cardiomyocyte stress responses (see below).

A number of studies have used high throughput miRNA profiling technologies to measure miRNA expression in control and failing human myocardium [21-25]. These studies primarily used ischemic cardiomyopathy (ICM) or idiopathic dilated cardiomyopathy (DCM) myocardium from hearts explanted at the time of transplantation, and therefore represent end stage heart disease. Selected findings are summarized in Table **1**. Thum *et al.* showed that microRNA expression in the failing myocardium shifts towards a fetal heart miRNA expression pattern [25]. mRNAs upregulated in failing hearts were enriched for predicted targets of downregulated miRNAs, and vica versa, suggesting that altered miRNA expression accounts in part for changes in the mRNA transcriptome observed in the failing human heart. We studied expression of 428 miRNAs in 67 human left ventricular samples belonging to control, ICM, DCM, and aortic stenosis (AS) groups [21]. Out of 87 confidently detected miRNAs, 43 were differentially expressed in at least one disease group. miRNA expression profiles were distinct between diagnostic groups and were able to classify samples by diagnosis with surprising accuracy. Differences in miRNA expression profiles between ICM and DCM were confirmed by Sucharov *et al.* [24]. Matkovich *et al.* profiled

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Table 1. Summary of Profiling Studies

Study	van Rooij *et al.*			Thum *et al.*		Ikeda *et al.*			Sacharov *et al.*		Matkovich *et al.*	
Study Description												
Species	M	M	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu
Ctrl	sham	Ntg	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Expt	TAC	CnA	HF[a]	DCM	fetal	ICM	DCM	AS	DCM	ICM	HF[b]	LVAD[c]
n (Ctrl v Disease)	1 pool of 3 samples		4 v 6	4 v 6	4 v 6	10 v 19	10 v 25	10 v 13	6 v 5	6 v 5	11 v 17	11 v 10
Platform	miRMAX microarray			mirVana microarray		In house, based on Luminex			LC Science outsource		Ncode microarray	
Differential Expression	Fold change > 1.5			Fold change > 1.5, p<0.05		ANOVA with post hoc test p< 0.05, FDR < 5%			t-test, p< 0.10		Fold change > 2.0, p<0.05	
Fold Change miRNA Expression												
miRNA	**TAC mice**	**CnA Tg mice**	**Human HF**	**Human HF**	**Fetal**	**DCM**	**ICM**	**AS**	**DCM**	**ICM**	**Human HF**	**Post-LVAD**
let-7a				1.6	2.4	1.1	1.1	1.0		down		
let-7b				2.2	1.6	1.4	1.2	1.1				
let-7c				2.2	2.9	1.3	1.2	1.2		down		
let-7d				2.9	4.0	1.1	1.1	1.0		down		
let-7d*						4.1	1.8	0.7				
let-7e				2.8	3.4	1.1	1.1	1.3				
let-7f				2.0	2.7	0.8	1.0	0.9		down	4.8	1.2
let-7g						0.9	1.0	0.9			4.7	1.4
let-7i											3.4	1.7
miR-1				2.9	3.2	0.6	0.8	0.7		down	15.2	2.4
miR-10a						0.8	1.2	0.2				
miR-10b	up	up		1.6	1.8	1.0	1.6	0.5	down			
miR-15a						0.9	1.1	1.0			3.9	1.5
miR-15b						1.7	1.3	1.7				
miR-16				0.4	2.9	1.0	1.0	1.0			5.1	1.7
miR-17-5p				0.5	3.7	0.8	0.9	0.9				
miR-17-3p				4.7	1.8							
miR-19a	up	up				0.4	0.8	0.4				
miR-19b				0.4	4.7	0.5	0.8	0.5				
miR-20a						0.8	0.9	0.8	down			
miR-20b						0.8	0.8	0.7				
miR-21	up	up	NC	5.0	10.9	0.8	1.0	0.7			2.7	1.1
miR-22				0.6	4.2	1.1	1.0	1.1	down		10.3	2.2
miR-23a	up	up				1.2	1.1	1.3	up		2.3	1.1
miR-23b	up	up		0.7	0.9	1.1	1.0	1.2				
miR-24	up	up	1.5	0.6	0.7	1.1	1.1	1.3			6.3	1.9

(Table 1) Contd…..

Fold Change miRNA Expression												
miRNA	**TAC mice**	**CnA Tg mice**	**Human HF**	**Human HF**	**Fetal**	**DCM**	**ICM**	**AS**	**DCM**	**ICM**	**Human HF**	**Post-LVAD**
miR-25	up	up										
miR-26a				2.0	3.6	1.1	1.1	1.0			2.7	1.7
miR-26b						0.8	1.0	0.8		up	8.8	1.4
miR-27a	up	up	NC	0.5	0.5	1.1	1.2	1.2			8.0	1.7
miR-27b	up	up				1.1	1.1	1.2			3.2	1.1
miR-28				1.5	1.7	0.9	0.9	0.9		up		
miR-29a				3.3	0.2	1.0	1.1	1.0			4.1	2.2
miR-29b				2.7	1.2	0.8	1.0	0.8			6.4	1.6
miR-29c	down	down	NC	2.2	0.3	0.8	1.0	0.8				
miR-30a-3p						0.9	0.9	0.9				
miR-30a-5p				0.5	0.8	0.9	1.0	0.9			3.1	1.1
miR-30b				0.3	0.9	1.0	0.9	0.9			12.0	2.3
miR-30c					0.8	1.0	1.0	1.0	down		3.3	1.3
miR-30d				0.4		1.1	1.0	1.1			3.0	1.2
miR-30e-3p						1.0	0.9	1.0				
miR-30e-5p				0.5	0.6	0.5	0.8	0.5				
miR-32				1.8	6.8							
miR-34b				2.0	1.4							
miR-92						0.9	0.8	0.8	down	down		
miR-93	down	down	NC			1.2	1.2	1.4				
miR-98				1.8	1.3	0.8	1.0	0.9				
miR-99a						1.1	1.1	1.2				
miR-99b						1.4	1.2	1.5				
miR-100						1.6	1.5	1.7	up	up		
miR-101						0.5	0.9	0.5				
miR-103						1.4	1.3	1.2			3.5	1.2
miR-106a						0.8	0.9	0.9				
miR-106b				1.6	1.8	1.0	1.1	1.1				
miR-107				0.6	0.9	1.1	1.0	1.0				
miR-125a				3.0	6.0	1.2	1.0	1.2				
miR-125b	up	up	1.3			1.4	1.2	1.4	up		4.0	2.2
miR-126	up	up		0.6	0.4	0.9	1.0	0.8			7.2	1.7
miR-126*				2.5	1.7	0.6	0.8	0.6				
miR-129				2.4	7.9							
miR-130a				2.5	8.8	1.3	1.2	1.2			3.1	1.4
miR-130b				0.6	0.2							

(Table 1) Contd…..

Fold Change miRNA Expression												
miRNA	**TAC mice**	**CnA Tg mice**	**Human HF**	**Human HF**	**Fetal**	**DCM**	**ICM**	**AS**	**DCM**	**ICM**	**Human HF**	**Post-LVAD**
miR-132				1.8	1.7							
miR-133a	down	down				1.0	1.0	1.0	down	down	2.6	1.5
miR-133b	down	down				1.0	1.0	1.0	down	down	3.9	1.7
miR-135a				0.7	0.7							
miR-136				0.5	0.7							
miR-139									down			
miR-140*						1.4	1.2	1.5				
miR-143						1.1	1.1	1.1			9.0	1.9
miR-145						1.2	1.1	1.3				
miR-146a						0.9	0.9	0.8				
miR-146b						0.8	0.8	0.6				
miR-148a				0.5	0.9							
miR-150	down	down	NC	0.4	9.9	1.4	1.0	1.2	down	down		
miR-151*						1.2	1.1	1.1				
miR-152						1.0	1.1	0.9				
miR-154	up	up										
miR-181a						1.7	1.3	1.7				
miR-181b	down	down	NC						up			
miR-182				0.4	0.8							
miR-185						1.0	0.9	1.0				
miR-186				0.5	0.5							
miR-191						1.4	1.3	1.6				
miR-191*						0.8	0.8	0.8				
miR-195	up	up	3			1.1	1.2	1.0	up	up	5.9	1.4
miR-196a				1.6	1.9							
miR-197									down			
miR-199a	up	up	2.1									
miR-199a*	up	up		0.5	0.5	1.7	1.7	1.3			5.6	1.9
miR-199b				2.2	1.5							
miR-200c				2.2	1.5							
miR-204				1.5	5.1							
miR-205				1.8	1.4							
miR-208				1.6	1.2	0.5	1.2	0.6				
miR-210	up	up		2.0	4.3							
miR-211				1.9	3.1							
miR-212				8.1	2.1							

(Table 1) Contd…..

Fold Change miRNA Expression													
miRNA	**TAC mice**	**CnA Tg mice**	**Human HF**	**Human HF**	**Fetal**	**DCM**	**ICM**	**AS**	**DCM**	**ICM**	**Human HF**	**Post-LVAD**
miR-213				2.6	2.5							
miR-214	up	up	1.6			2.8	2.1	2.1				
miR-217	up	up										
miR-218	up	up		0.7	0.9							
miR-221									down	down		
miR-222						0.6	0.6	0.9		down		
miR-224										down		
miR-296				1.7	0.2							
miR-299-5p				0.3	18.4							
miR-302a				1.5	1.2							
miR-302b*				0.6	0.7							
miR-302c*				0.3	0.6							
miR-320				3.4	3.9	1.4	1.4	1.4				
miR-330	up	up										
miR-331				1.8	2.4							
miR-335						0.8	0.9	0.8				
miR-339				0.7	0.9							
miR-340				2.8	6.7							
miR-342				0.5	0.7	1.5	1.2	1.3		up		
miR-351	up	up										
miR-361						1.1	1.0	1.2				
miR-365				2.8	1.0	1.3	1.1	1.1				
miR-367				1.7	1.7							
miR-372				2.3	1.1							
miR-373				2.1	1.8							
miR-374						0.7	1.0	0.7				
miR-377				1.7	2.7							
miR-378											3.6	1.2
miR-381				2.1	6.7							
miR-382				1.7	1.9				up			
miR-422b						0.9	0.8	1.0	down	down		
miR-423				5.1	4.6							
miR-423*						1.4	1.2	1.4				
miR-424				3.0	12.6	0.8	1.3	0.7				
miR-429				1.9	1.2							
miR-432				1.5	2.2							

(Table 1) Contd…..

Fold Change miRNA Expression												
miRNA	**TAC mice**	**CnA Tg mice**	**Human HF**	**Human HF**	**Fetal**	**DCM**	**ICM**	**AS**	**DCM**	**ICM**	**Human HF**	**Post-LVAD**
miR-451						1.1	1.3	0.9				
miR-452*				0.5								
miR-483									down			
miR-483*						1.0	1.0	1.1				
miR-484										down		
miR-486									down	down		
miR-494				0.6	0.7							
miR-495				0.7		0.7	0.7	0.9				
miR-497				0.4	0.6							
miR-499				0.2		0.7	0.7	0.9			3.6	1.2
miR-500				1.6								
miR-507				0.6	0.9							
miR-512-5p				0.7	1.0							
miR-515-5p				0.4	0.9							
miR-520c				1.9	2.2							
miR-520d*				0.5	0.7							
miR-520h				0.7								
miR-523				0.5	3.9							
miR-525*				2.0	1.1							
miR-526b				0.5	0.7							
miR-526b*				0.3	0.5							
miR-594									down	down		
miR-638											2.8	0.8

TAC, thorasic aortic constriction; Ntg, non-transgenic control; CnA, activated calcineurin transgenic; HF, heart failure; NF, non-failing control; ICM, ischemic cardiomyopathy; DCM, idiopathic dilated cardiomyopathy; AS, aortic stenosis; LVAD, left ventricular assist device; NC, no change
[a]Heart failure class not specified;[b]LVAD = 4 ICM, 6 DCM; [c]LVAD = 4 ICM, 6 DCM

(pink)	significantly upregulated by each criteria
(blue)	significantly downregulated by each criteria
number	no statistically significant difference
empty	Not reported, or data not available

miRNA expression in non-failing, failing, and mechanically unloaded failing human myocardium, and found that the combination of miRNA and mRNA expression profiles was best able to assign accurate diagnostic labels to samples [23]. Interestingly, while mechanical unloading only normalized a small minority of mRNA transcripts, it normalized nearly all miRNAs, suggesting that miRNAs are more sensitive indicators of the functional status of end-stage cardiomyopathic myocardium. Collectively, these miRNA profiling studies show that miRNA signatures differ by heart disease etiology, providing proof of principle that miRNAs may have therapeutic use as informative biomarkers in heart disease. Further work using myocardial samples with clinical followup data will be required to determine if miRNA profiles will be useful for predicting prognosis or treatment response.

For clinical use, circulating biomarkers have substantial practical advantages compared to myocardial biomarkers. Recent studies have shown that circulating miRNAs may be useful diagnostic biomarkers. The cardiac-restricted miRNAs miR-1, miR-133, miR-499, and miR-208a were significantly increased in plasma as soon as 1 hour after myocardial infarction [26], suggesting that these miRNAs may be useful in the diagnosis of acute myocardial infarction. miRNA profiling of plasma from control and heart failure patients identified six miRNAs upregulated in heart failure [27], providing proof of concept that plasma miRNA signatures in may contain diagnostically and prognostically useful information in heart failure.

At the level of specific miRNAs, most studies showed differential expression of miR-21, miR-23a, miR-24, miR-26b, miR-27a, miR-125b, miR-195, miR-199a-3p in diseased compared to control myocardium (Table **1**). It can therefore be concluded with reasonable confidence that these miRNAs are differentially expressed in late stage human heart failure. Each study identified many other differentially expressed miRNAs, but inconsistency between studies makes it more difficult to reach conclusions on whether these miRNAs are differentially expressed. Factors that have limited reproducibility are (a) inherent variability in disease and control samples, related to diagnosis, disease stage and myocardial sampling; (b) variable preservation of disease and particularly control myocardium; (c) technical differences between miRNA assay technologies, and (d) small sample numbers and variable statistical methods. Future profiling studies with larger numbers of carefully phenotyped, well-preserved, and matched disease and control samples, rigorous statistical design, and "gold-standard" quantitative assay platforms (e.g. qRTPCR or deep sequencing) will be needed to elucidate global miRNA expression changes in heart disease.

While cardiomyocytes are the signature lineage of the myocardium, the heart also contains several other cell types (endothelial cells, smooth muscle cells, fibroblasts) that are critical for heart function and stress responses. These other cell lineages are also enriched for characteristic miRNAs or may contribute to measured changes in miRNA expression. For example, endothelial cells are highly enriched for miR-126 [28-30], and cardiac fibroblasts are highly enriched for miR-21 and miR-29 compared to myocytes [31, 32]. An important limitation of published profiling studies has been the cellular heterogeneity of myocardial samples. This heterogeneity impairs detection of expression differences within one cell type, makes it difficult to pinpoint the cell type(s) with altered expression, and can lead to inaccurately inferring that expression differences caused by different proportions of cell types represent altered expression within one cell type. This limitation can be overcome in experimental models using cellular fractionation approaches, but these approaches are more difficult to apply to archived human myocardial samples.

MIRNA FUNCTION IN THE DEVELOPING AND ADULT HEART

To test the global requirement of miRNA in the developing and adult heart, the essential miRNA processing genes *Dicer* and *Dgcr8* have been inactivated in cardiomyocytes, resulting in marked reduction of levels of all miRNAs. Cardiomyocyte-restricted inactivation of *Dicer* by *Nkx2-5^{Cre}*, expressed in cardiac progenitors as well as differentiated cardiomyocytes, resulted in death from heart failure by embryonic day 12.5. Mutant hearts exhibited poorly developed ventricular myocardium and abnormal gene expression [33]. This suggests that miRNAs are globally required for normal cardiomyocyte differentiation and proliferation. Additionally, these data suggest that although miRNAs promote cardiomyocyte specification [30], they are not absolutely required for this process. Alternatively, *Dicer* inactivation may not reduce levels of critical miRNAs rapidly enough to unmask the requirement for miRNAs in cardiomyocyte specification. Later stage inactivation of *Dicer* by cardiomyocyte-specific *Myh6-Cre* resulted in perinatal lethal cardiomyopathy characterized by misexpression of contractile proteins and sarcomere disarray [34]. Adult-stage *Dicer* inactivation by *Myh6-MerCreMer* (tamoxifen-induced Cre recombinase) similarly resulted in lethal dilated cardiomyopathy, confirming that miRNAs are required in the adult heart to maintain normal function [35].

Dicer has roles outside of miRNA processing, so the specificity of these findings to loss of miRNA was uncertain. This issue was recently addressed by loss of function studies of *Dgcr8*. *Dgcr8* and *Dicer* are each required for distinct, independent steps in miRNA biogenesis. Perinatal inactivation of *Dgcr8* by *muscle creatine kinase (MCK)-Cre* caused a lethal dilated cardiomyopathy, with median survival of 21 days [19]. The finding that ablation *Dgcr8* results in a similar cardiomyopathic phenotype strongly reinforces the essential role of microRNA regulation in maintaining cardiac function. An additional potential benefit of the *Dgcr8* loss of function approach is that, unlike Dicer knockouts, *Dgcr8* knockouts retain the ability to process short hairpin RNA intermediates expressed from transgenes into mature miRNAs. Thus, rescue experiments in the *Dgcr8* knockout background may identify minimal miRNA requirements for maintenance of cardiac function.

While disruption of miRNA biogenesis has revealed global requirements for miRNAs in the heart, this approach is unable to pinpoint roles for specific miRNA-mRNA interactions that are crucial for heart development and function. Such mechanistic insights have come from gain and loss of function studies of specific miRNAs *in vitro* and *in vivo* (Table **2**). These studies are summarized below.

MUSCLE-SPECIFIC MIRS: MIR-1 AND MIR-133

miR-1/206 and miR-133a/133b are expressed in muscles from 3 distinct primary transcripts: miR-1-1/miR-133a-2 on human chromosome 20, miR-1-2/miR-133a-1 on human chromosome 18, and miR-206/miR-133b on human chromosome 6. miR-206, which differs from miR-1 by only 4 nucleotides, and miR-133b are expressed only in skeletal muscle, while miR-1-1/miR-133a-2 and miR-1-2/miR-133a-1 are expressed in both cardiac and skeletal muscle. miR-1 expression is notably high, accounting for 40% of all mature miRNAs in the heart [19].

As anticipated by their selective expression in muscle cells, miR-1 and miR-133 play crucial roles in myocyte

Table 2. microRNA Function and Target Genes

<table>
<tr><th></th><th>Proliferation</th><th>Differentiation</th><th>Hypertrophy</th><th>Apoptosis</th><th>Fibrosis</th><th>Angiogenesis</th><th>Arrhythmia</th></tr>
<tr><td rowspan="2">miR-1</td><td></td><td>Promote methoderm and cardiac lineage
DLL-1 (green)</td><td></td><td>HSP60
HSP70
BCL2 (green)</td><td></td><td></td><td>GJA1
CKNJ2
Irx5 (green)</td></tr>
<tr><td>HAND2 (red)</td><td></td><td>RASGAP
CDK9
RHEb
CALM1
CALM2
MEF2A
• GATA4 (red)</td><td></td><td></td><td></td><td>HCN2
HCN4 (red)</td></tr>
<tr><td rowspan="2">miR-21</td><td></td><td></td><td></td><td></td><td>SPRY1
PTEN (green)</td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td>PDCD4 (red)</td><td></td><td></td><td></td></tr>
<tr><td>miR-23a</td><td></td><td></td><td>MURF1 (red)</td><td></td><td></td><td></td><td></td></tr>
<tr><td>miR-29</td><td></td><td></td><td></td><td></td><td>COL1A1
COL1A2
COL3A1
FBN1
ELN (red)</td><td></td><td></td></tr>
<tr><td>miR-126</td><td></td><td></td><td></td><td></td><td></td><td>SPRED1
PI3KR/p85b (green)</td><td></td></tr>
<tr><td rowspan="2">miR-133</td><td>SRF
Cyclin D2 (green)</td><td>Promote mesoderm lineage (green)</td><td>KO: no hypertrophy phenotype</td><td></td><td></td><td></td><td>ERG,
KCNQI
KCNEI (green)</td></tr>
<tr><td></td><td>Suppress cardiac lineage (red)</td><td>Antagomir: miR-133 suppresses hypertrophy (red)</td><td>Caspase 9 (red)</td><td>CTGF (red)</td><td></td><td>HCN2 (red)</td></tr>
<tr><td rowspan="2">miR-208a</td><td></td><td></td><td>Promotes slow muscle gene pgm (green)</td><td></td><td></td><td></td><td>Gata4
• Hopx (↓ in null)
• Gja5 (↓ in null) (green)</td></tr>
<tr><td></td><td></td><td>THRAP1
Myostatin (red)</td><td></td><td></td><td></td><td></td></tr>
</table>

green promote via indicated target genes

red suppress via indicated target genes

• indirect

specification, differentiation, growth, survival, and signaling. In differentiating C2C12 skeletal myoblasts, miR-1 and miR-133 were shown to regulate progenitor cell differentiation and proliferation [18]. miR-1 promoted skeletal muscle differentiation by targeting *histone deacetylase 4* (*HDAC4*), a transcriptional repressor of muscle gene expression. Interestingly, miR-133 promoted progenitor cell proliferation and antagonized differentiation by downregulating the key myogenic transcription factor SRF. Similar results were observed during cardiomyocyte specification in human and murine ES cells. miR-1 and miR-133 overexpression in ES cells promoted mesodermal differentiation [30] and acted to repress non-muscle gene expression and fate. miR-1 further promoted differentiation of mesodermal cells into cardiac progenitors, while miR-133 acted in an opposing manner [30]. miR-1 activity was mediated by translational repression of the Notch ligand *Dll-1*. These data demonstrate that miR-1 and miR-133 act to regulate myogenic progenitor cell specification and differentiation.

miR-1 also regulates cardiomyocyte proliferation and differentiation. miR-1 directly represses *Hand2*, a basic helix-loop-helix transcription factor critical for cardiac morphogenesis and normal ventricular growth. Overexpression of miR-1 reduced cardiomyocyte proliferation and caused myocardial hypoplasia [16]. Conversely, partial miR-1 loss of function, due to miR-1-2 knockout, resulted in increased cardiomyocyte proliferation and upregulation of *Hand2* [33]. Approximately half of miR-1-2 knockout mice died in late gestation with ventricular septal defects and likely primary myocardial dysfunction, while surviving mutant mice displayed heterogeneous phenotypes. Some developed heart failure, while the remainder maintained normal cardiac function but were susceptible to sudden death, presumably from lethal arrhythmias. miR-1-2 knockout mice showed abnormal cardiac conduction, which was linked to miR-1 repression of *Irx5*, a transcriptional regulator of cardiac channel gene expression. Increased expression of miR-1 has also been linked to arrhythmogenesis, with the gap junction

gene *Gja1* (connexin 43) and the potassium channel gene *Kcnj2* implicated as direct, responsible miR-1 targets [36]. The phenotypes of miR-1-1 and miR-1-1/miR-1-2 loss of function mice has yet to be reported.

Under stress, cardiomyocytes increase in size (hypertrophy), a process that is initially compensatory but that ultimately predisposes to heart failure. Overexpression of miR-1 by adenovirus was sufficient to prevent cellular hypertrophy both *in vitro* and *in vivo* [15, 37, 38]. Calcium signaling is a key mediator of cardiomyocyte hypertrophy, and miR-1 negatively regulated two central regulators of calcium signaling, calmodulin and Mef2a [15]. Three independent transcripts (*Calm1-3*) encode identical calmodulin polypeptide. The two major cardiac transcripts, *Calm1* and *Calm2*, were both negatively regulated by miR-1, and miR-1 downregulation was associated with calmodulin upregulation in a heart failure caused by transgenic calcineurin expression. Transgenic calmodulin overexpression to a similar degree caused cardiac hypertrophy and failure [39], suggesting that calmodulin levels are important regulators of calcium signaling and that dysregulation of miR-1 and calmodulin contribute to the heart failure phenotype of calcineurin overexpressing mice. MEF2A and GATA4 transcription factors are key regulators of cardiac gene expression downstream of calcium-calmodulin signaling. Expression of *Mef2a* was directly and *Gata4* indirectly downregulated by miR-1 [15]. These data suggest that miR-1 attenuates cardiac hypertrophy by negatively regulating calcium signaling at multiple levels. Other identified miR-1 targets are genes encoding the Ras GTPase-activating protein (RasGAP), cyclin-dependent kinase (Cdk9), and Ras homolog enriched in brain (Rheb) [37].

Expression of miR-1 has been reported to be both up- and down-regulated in both high throughput profiling studies of human heart disease (Table **1**) and in more focused studies on miR-1 [21, 36-38]. miR-1 expression has been found to be variably increased or decreased in high throughput studies of human heart disease (Table **1**). Yang *et al.* reported that miR-1 is upregulated in hearts explanted from individuals with coronary artery disease, and in the ischemic zone of rats after experimental myocardial infarction [36]. On the other hand, miR-1 was reported to be downregulated in human hypertrophic cardiomyopathy and in atria dilated secondary to mitral stenosis [38]. In murine heart failure due to aortic constriction, transgenic calcineurin overexpression, transgenic Akt overexpression, and physiologic, exercise-induced hypertrophy, miR-1 was down-regulated [21, 37, 38].

miR-133 has also been shown to have key roles in regulating cardiac development and function. *In vitro* and *in vivo* adenoviral-mediated overexpression of miR-133 inhibited agonist-induced hypertrophy [38]. Consistent with this finding, miR-133 loss of function by infusion of chemically modified antisense oligonucleotide directed against miR-133 (“antagomir” [40]) and by adenoviral expression of tandemly repeated miR-133 antisense sequences (miRNA “sponge” [41]) reduced miR-133 levels and caused cardiomyocyte hypertrophy [38]. These results suggested that miR-133 regulates cardiomyocyte hypertrophy. The Rho family members *RhoA* and *Cdc42*, and *Nelfa*, a negative regulator of RNA polymerase II, were implicated as direct miR-133 targets that mediate the inhibitory effects of miR-133 on cardiomyocyte hypertrophy [38].

However, genetic loss of miR-133 function by double knockout of both cardiac isoforms (miR-133-a-1 and miR-133-a-2) did not show evidence of cardiomyocyte hypertrophy [42]. The discrepancy between genetic loss of function and antagomir-mediated loss of function is incompletely understood but important to note. Potential causes are off-target effects of antagomirs, genetic compensation for constitutive genetic loss of function, and unrecognized functional activity of miRNA intermediates that are affected by genetic but not antagomir loss of function approaches. miR-133 transgenic gain of function (*Mhy6-miR-133*) increased mature miR-133 levels by ~13 fold, but did not affect heart size or function at baseline or after aortic constriction [43], again consistent with the conclusion that miR-133 does not regulate cardiomyocyte hypertrophy *in vivo*.

While miR-133 loss of function did not influence cardiomyocyte hypertrophy, there were abnormalities of cardiac structure and function [42]. Mutant embryos exhibited hypoplasia of the compact myocardium and underdevelopment of the apical portion of the interventricular septum. Roughly 60% of mice were dead by postnatal day 1, with dilated, engorged atria and large VSDs at the apex and near the atrioventricular valves. Mice that survived to adulthood lacked VSDs and exhibited severe ventricular dysfunction. Mutant mice showed sarcomere fragmentation and disorganization, and upregulation of markers of cardiac stress. Consistent with miR-133 downregulation of SRF [18], SRF was upregulated in miR-133 knockouts [42]. SRF is also an important driver of smooth muscle gene expression, and miR-133 knockout was associated with increased expression of several SRF-regulated smooth muscle genes [42]. miR-133 knockout mice exhibited aberrant cardiomyocyte proliferation. The cell cycle regulatory gene *cyclin D2* was identified as a direct target of miR-133, and was upregulated in miR-133 double knockouts [42].

Myocardial fibrosis contributes to cardiovascular insufficiency in heart disease. miR-133 plays an important role in inhibiting myocardial fibrosis, as miR-133 knockout mice developed severe myocardial fibrosis and increased cardiomyocyte apoptosis [42]. Cardiac fibrosis and cardiomyocyte apoptosis in miR-133 knockouts may also be stimulated by the pro-fibrotic cytokine connective tissue growth factor (CTGF), which is negatively regulated by miR-133 [44]. Consistent with an anti-fibrotic role of miR-133, *Myh6-miR-133* transgenic mice were resistant to myocardial fibrosis and cardiomyocyte apoptosis induced by pressure overload [43]. However, miR-133 overexpressing mice did not exhibit altered expression of CTGF (or SRF or Cyclin D2).

MYOMIRS: MIR-208A/208B/499

Cardiomyocytes express two major myosin heavy chain genes *Myh6* (myosin heavy chain) and *Myh7* (myosin heavy chain), in addition to low levels of the minor myosin heavy chain gene *Myh7b*. The encoded proteins have distinct contractile properties, and the balance of *Myh6* and *Myh7* expression is a determinant of cardiac contractility and myocardial energy consumption [45]. Introns within *Myh6*, *Myh7*, and *Myh7b* encode miR-208a (also known as miR-

208), miR-208b, and miR-499, respectively. These closely related "myomirs" are powerful regulators of myosin gene expression and cardiac stress responses, and thereby confer these sarcomeric genes with important regulatory properties.

miR-208a knockout mice are viable and have normal cardiac size and function at baseline up to 5 months of age, at which point they exhibit a mild decline in cardiac function [46]. In response to pressure overload, miR-208a knockout hearts developed a greater degree of ventricular dysfunction than wild-type controls. Remarkably, miR-208a knockout hearts were resistant to cardiac hypertrophy and fibrosis induced by cardiac stress (aortic constriction and transgenic calcineurin overexpression). Expression of *Myh7*, normally strongly upregulated by cardiac stress, was unchanged in miR-208a KO hearts. Induction of other stress-responsive genes, such as *Nppa* and *Nppb*, was preserved in miR-208a KOs, suggesting specific dysregulation of *Myh7*. Conversely, 4-fold overexpression of miR-208a was sufficient to elicit cardiomyocyte and cardiac hypertrophy [47]. Cardiac systolic function was mildly diminished in miR-208a Tg hearts, and *Myh7* was markedly upregulated. Thyroid hormone is known to promote cardiac hypertrophy, and the thyroid hormone receptor (TR) directly represses *Myh7* expression. miR-208a was shown to directly inhibit expression of the TR coregulator THRAP1 [40, 41]. Loss of miR-208a caused increased THRAP1, enhancing TR-mediated repression of *Myh7*. miR-208a also regulates myostatin, a powerful regulator of skeletal and cardiac muscle growth [47]. Another target of miR-208a is the key cardiac transcription factor GATA4. The *Gata4* 3' UTR contains an evolutionarily conserved miR-208a seed match sequence that responds to miR-208a *in vitro*, and GATA4 levels were upregulated in miR-208a knockout mice [47].

Cardiac conduction was also disturbed in miR-208a transgenic mice. These mice had first and second degree atrioventricular block. Interestingly, miR-208a knockout mice lacked P-waves on surface electrocardiograms, suggesting that they may have atrial fibrillation. The direct molecular targets of miR-208a responsible for these conduction abnormalities have not been identified. Gap junctions are important for cardiac conduction. The gap junction gene *Gja5* (Cx40) appears to be indirectly downstream of miR-208a, because *Gja5* was downregulated in miR-208a null mice. However, it was not affected in miR-208a Tg hearts. *Gja5* is transcriptionally regulated by the transcriptional co-factor *Hopx* [48], and *Hopx* was not detectable in miR-208a null mice. The direct molecular targets through which miR-208a mediates these effects is not known.

Further investigation of myomirs has eludicated a regulatory hierarchy that governs muscle myosin content and muscle gene expression [49]. *Myh6*/miR-208a are required for expression of the slow muscle myosins *Myh7* and *Myh7b*, as well as their myomirs miR-208b and miR-499. Loss of miR-208a blocked expression of *Myh7*/miR-208b and ectopically activated fast skeletal muscle genes. Transgenic expression of miR-499 in miR-208a null mice normalized expression of *Myh7*/miR-208b and fast skeletal muscle genes. These data suggest that miR-499 is a downstream mediator of miR-208a in cardiac sarcomere gene expression. Genetic deletion of either miR-499 or miR-208b alone did not affect survival, mice displayed no overt abnormalities, and cardiac expression of *Myh7* was normal [49]. This may be due to functional redundancy, as miR-499 and miR-208b exhibit functionally redundancy in skeletal muscle [49]. Sox6, Pur , and Sp3, transcriptional repressors previously reported to repress *Myh7*, were validated as miR-208a targets. Consistent with a role downstream of miR-208a, transgenic expression of *Sox6* to levels comparable to those observed in miR-208a knockout mice extinguished *Myh7b* and miR-499 expression [49].

In adult rodent myocardium *Myh6* is the major myosin heavy chain, while in humans *Myh7* predominates. Accordingly, miR-208b is likely to be the major myomir in the adult heart, as suggested by a survey of miRNA expression [50]. The hierarchy of myomir regulation of myosin heavy chain expression in human cardiomyocytes is an important unresolved question. In human heart disease, *Myh6* is downregulated while *Myh7* is upregulated [45], but interestingly expression of miR-208 family members has not been reported to vary in human heart disease.

MIR-21 AND MIR-29: REGULATORS OF CARDIAC FIBROSIS

Myocardial fibrosis is an important complication of most forms of human heart disease. As outlined above, myocyte specific miRNAs influence myocardial fibrosis, presumably by regulating cardiomyocyte apoptosis and secretion of pro-fibrotic factors. miRNAs expressed in cardiac fibroblasts also regulate myocardial fibrosis. miR-21 and miR-29 are two such miRNAs that have attracted interest because of important roles in this process. miR-21 and miR-29 are widely expressed miRNAs that are highly enriched in non-myocytes (presumably cardiac fibroblasts) compared to myocytes [31, 32]. miR-21 is upregulated in most, but not all, miRNA profiling studies of experimental and human heart disease (Table **1**). miR-21 upregulation was confined to cardiac interstitial cells in an experimental heart failure model [31]. There has not been agreement on miR-29 expression in human heart disease (Table **1**). In myocardial samples from the border zone of experimental and human myocardial infarction, all three miR-29 family members (miR-29a/29b/29c) were downregulated compared to remote myocardium.

Upregulation of miR-21 in diseased cardiac fibroblasts has been shown to promote cardiac fibrosis [31]. Pressure overload is a potent stimulus for myocardial fibrosis. The fibrosis induced by pressure overload and chronic isoproterenol infusion was markedly attenuated by miR-21 antagomir knockdown, as was myocardial, cardiomyocyte hypertrophy, and ventricular dysfunction. Perhaps most remarkably, myocardial fibrosis, hypertrophy, and systolic dysfunction established by pressure overload were partially regressed by miR-21 antagomir administered three weeks after placement of the aortic band. Apoptosis was lower in cardiac fibroblasts isolated from failing compared to non-failing myocardium, and this difference was dependent upon miR-21 as well as ERK activation. miR-21 mediated resistance to apoptosis was attributed to downregulation of *Spry1*, a potent inhibitor of RAS-ERK signaling. Other identified targets of miR-21 in cardiac fibroblasts are PTEN, an inhibitor of Akt signaling, which regulates the matrix meta-

lloprotease MMP-2, an important regulator of cardiac extracellular matrix remodeling [51].

There are conflicting reports on the activity of miR-21 in cardiomyocytes. Thum *et al.* reported that miR-21 gain and loss of function in cardiomyocytes did not influence morphology or size of cardiomyocytes in culture or in mice with cardiomyocyte-specific miR-21 overexpression [31]. On the other hand, miR-21 overexpression in cultured cardiomyocytes was reported to cause long, slender outgrowths and downregulation of *Spyr2*. [52]. miR-21 has also been reported to protect cardiomyocytes for apoptosis, *via* the target gene *Pdcd4* [53], and to inhibit cardiomyocyte hypertrophy [54].

Down-regulation of miR-29 in border zone myocardium has been implicated in contributing to myocardial fibrosis [32]. Computationally predicted miR-29 targets included an unexpectedly high number of fibrosis-related mRNAs, including collagens, metallopeptidases, and integrins, and several of these fiborsis-related targets, including collagens Col1A1, Col1A2, and Col3A1, were biologically validated. miR-29b antagomir knockdown in mice increased expression of these collagens in the liver and heart. miR-29b overexpression in cultured cardiac fibroblasts diminished collagen expression.

MIR-126: REGULATOR OF ANGIOGENESIS

Cardiomyocytes are dependent on a rich vascular network, maintenance of which is dependent upon complex signaling between cardiomyocytes, cardiac interstitial cells, smooth muscle cells, and endothelial cells. The balance of proangiogenic and antiangiogenic signaling determines vascular homeostasis and new vessel growth following ischemic events. Recent work has identified multiple miRNAs that regulate angiogenesis. Here we highlight the role of the only known endothelial-specific miRNA, miR-126 [28, 29].

miR-126 is expressed from an intron of an endothelial-restricted host gene, *Egfl7*. Although *Egfl7* knockout had been shown to have angiogenic defects and loss of vascular integrity [55], more precise gene targeting experiments that separately caused *Egfl7* or miR-126 loss of function showed that this phenotype is due to loss of miR-126 rather than *Egfl7* [56]. miR-126 knockout mice, and miR-126 morpholino treated zebrafish, exhibited loss of vascular integrity [29, 56, 57]. Murine miR-126 knockout reduced angiogenesis and survival after myocardial infarction. miR-126 enhanced the response of endothelial cells to vascular endothelial growth factor (VEGF), at least in part by downregulating two powerful inhibitors of VEGF signaling, Sprouty-related protein SPRED1 and regulatory subunit 2 of phosphatidylinositol 3-kinase (PI3KR/p85b) [29, 57].

Interestingly, following myocardial infarction miR-126 was highly downregulated in both the myocardial infarct border zone and in the remote myocardium, compared to sham operated myocardium [32]. miR-126 was also downregulated in non-myocytes isolated from the severely hypertrophied myocardium of calcineurin transgenic mice [15]. These data suggest that miR-126 is dynamically regulated in response to cellular context, even at locations remote from the injury.

OTHER MIRNAS IMPLICATED IN CARDIAC PATHOLOGY

A number of other miRNAs have been implicated in cardiac disease pathogenesis. miR-23a expression was upregulated in most profiling studies of human heart disease (Table **1**). Activation of the calcium-dependent calcineurin-NFAT pathway, a key mediator of cardiac hypertrophy [58], upregulated miR-23a. Overexpression of miR-23a was sufficient to drive cardiomyocyte hypertrophy [22], and antagomir-mediated loss of miR-23a activity blocked calcineurin-mediated hypertrophy [58], at least in part by targeting the anti-hypertrophic factor MuRF1 [58]. Other miRNAs that have been implicated in regulated cardiac hypertrophy are miR-24, miR-195, miR-199a, and miR-214 [22]. miR-143 and miR-145 are co-transcribed miRNAs expressed in smooth muscle cells [59]. These miRNAs were downregulated in injured or atherosclerotic vessels and target key smooth muscle transcriptional regulators, including *Klf4*, *myocardin*, and *Elk1* to promote differentiation and inhibit proliferation of smooth muscle cells. These data suggest that miR-143 and miR-145 play a role in regulating smooth muscle responses in conditions such as atherosclerosis.

FUTURE DIRECTIONS

In a relatively brief time, investigations into miRNAs have revealed a previously unappreciated layer of gene regulation. Cardiac miRNAs have been shown to be powerful modifiers of cardiac gene expression, both by their pervasive effects on numerous target genes, and in some cases by their large, switch-like effects on key gene expression programs. However, we have likely only scratched the surface in understanding how miRNAs participate in cardiovascular disease pathogenesis.

Elucidation of mechanisms underlying miRNA function rely on identification of miRNA-mRNA interactions, a process which currently depends heavily on computational predictions. Development of empirical methods to identify these interactions in an unbiased manner will be an important advance. Also needed are improved methods for validating miRNA-mRNA interactions *in vivo*, to show that identified interactions are physiologically meaningful rather than *in vitro* artifact.

The ability of a single small RNA species to influence expression of hundreds to thousands of targets and to profoundly influence cellular phenotype is at once a strength and a potential pitfall of miRNAs as therapeutics. *In vivo* delivery of highly effective miRNA antagonists is currently feasible, and *in vivo* delivery of effective miRNA mimetics is likely on the immediate horizon. However, honing the activity of these powerful agents and predicting and limiting their potentially pleiotropic effects will be a major challenge in translating the promise of miRNAs to the clinical realm.

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